Sex ratio bias leads to the evolution of sex role reversal in honey locust beetles

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Abstract: The reversal of conventional sex roles was enigmatic to Darwin, who suggested that it may evolve when sex ratios are female biased [1]. Here we present direct evidence confirming Darwin's hypothesis. We investigated mating system evolution in a sex-role-reversed beetle (Megabruchidius dorsalis) using experimental evolution under manipulated sex ratios and food regimes. In female-biased populations, where reproductive competition among females was intensified, females evolved to be more attractive and the sex roles became more reversed. Interestingly, female-specific mating behavior evolved more rapidly than male-specific mating behavior. We show that sexual selection due to reproductive competition can be strong in females and can target much the same traits as in males of species with conventional mating systems. Our study highlights two central points: the role of ecology in directing sexual selection and the role that females play in mating system evolution.

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Sex-ratio bias leads to the evolution of sex-role reversal in honey locust beetles

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Summary: The reversal of conventional sex-roles was enigmatic to Darwin, who suggested that it may evolve when sex ratios are female-biased [1]. We present the first direct evidence confirming Darwin's hypothesis. We investigated mating system evolution in a sex-role reversed beetle (*Megabruchidius dorsalis*) using experimental evolution under manipulated sex ratios and food regimes. In female-biased populations, where reproductive competition among females was intensified, females evolved to be more attractive and the sex-roles became more reversed. Interestingly, female-specific mating behaviour evolved more rapidly than male-specific mating behaviour. We show that sexual selection due to reproductive competition can be strong in females and can target much the same traits as in males of species with conventional mating systems. This highlights the central role that females play in mating system evolution.

Results and Discussion:

"If we might assume that the females have become much more numerous than the males […], then it is not improbable that the females would have been led to court the males, instead of being courted by them."

Charles Darwin, 1871 [1]

The conventional animal mating system, with showy males and choosy females, is reversed in a minority of taxa. In such sex-role reversed mating systems, females instead court males and are sometimes equipped with elaborated ornaments [2] while males are the more discriminating sex and may reject female mating attempts [3]. Darwin [1] postulated that such reversals are the evolutionary result of female-biased operational sex ratios (OSR), where females compete for access to resources provided by males [4]. There is some comparative evidence for this tenet [5-8] and a few studies of plasticity in sex-roles have suggested a key role for OSR [9, 10]. Here, we employ experimental evolution in an insect to provide the first direct test of Darwin's fundamental prediction: that sex-role reversal evolves under female-biased OSR.

In the honey locust beetle (*Megabruchidius dorsalis*), sex-roles in courtship are reversed compared to other seed beetles: females search for males, actively initiate mating and court males vigorously, whereas males often reject female mating attempts [11-13]. Male ejaculates provide females with essential nutrients that increase females' fecundity and longevity [14], and females that secure more

matings live longer and produce more offspring [8, 11, 15]. In contrast, matings carry substantial direct costs to males [13, 14] and males prefer to mate with females that perform more vigorous courtship displays [11, 13].

We allowed replicated honey locust beetle populations to evolve for 19 generations under two adult sex ratios (female-biased, 1:5, or male-biased, 5:1) and food regimes (abundant food or no food during adulthood) using a full factorial design (Experimental procedures). Due to male investment in ejaculates, the inter-mating interval is longer in male than in female honey locust beetles [14, 15], so the potential reproductive rate [8] is somewhat lower in males. We predict competition between females over access to males to be intensified under female-biased and greatly relaxed under male-biased sex ratios [16-18]. Our experimental design enabled us to directly measure the evolution of female courtship behavior as a response to increased and relaxed sexual selection in females. Inclusion of a food treatment allowed us to assess whether male-provisioned resources can compensate for normal feeding, in which case we expect the evolutionary effects of sexual selection in females to vary with resource availability. Following post-selection rearing under common garden conditions, we quantified male-female courtship interactions and the fitness effects of mating using replicated sex-specific mating system assays that paired beetles from the selection lines with standard reference individuals of the opposite sex.

Females that evolved under female-biased OSR (elevated reproductive competition) significantly outperformed females from male-biased lines in courtship and mating success when paired with standard reference males. Females from female-biased lines made earlier contact with prospective mates, started courting males sooner, showed higher courtship intensity (total number of turns, number of turns in 1st courtship bout, time to first courtship) and achieved successful copulation sooner and after fewer male pre-copulatory mountings (Figure 1, Table 1). Thus, as predicted, increased sexual selection in females led to the evolution of a more sex-role reversed mating system: females courted males more intensely and at a higher rate. As a result, these females were effectively more attractive to reference males, who more readily accepted them as mates.

In contrast to the marked evolutionary response seen in female behavior, male-specific traits showed little evolution as a result of altered reproductive competition (Table 2). Males from male-biased lines were as slow to mate as those from female-biased lines. Multivariate analyses of variance of our eight behavioral mating system variables collectively provided evidence for overall evolution in females (Pillai trace = 2.23, $F_{24,18}$ = 2.18, P = 0.047) but not in males (Pillai trace = 1.99, $F_{24,18}$ = 1.49, P = 0.193). Further, Bartlett's sequential residual root test revealed two significant orthogonal multivariate evolutionary response dimensions in females (root 1-3: χ^2_{24} = 43.42, P = 0.009; root 2-3: χ^2_{14} = 23.72, P = 0.049) but none in males (root 1-3: χ^2_{24} = 35.23, P = 0.065; root 2-3: χ^2_{14} = 14.67, P = 0.401). We conclude that alteration of

the sexual selection regime affected the evolution of sex-specific behaviors more in females than in males.

Several studies have proposed that sexual selection should differ fundamentally between the sexes due to sex-specific investment trade-offs [2, 19]. This is often held to be a consequence of anisogamy and the larger cost of egg production relative to sperm production [20, 21]. However, this inference is problematic when males provide females with direct benefits that impose similar or even greater costs relative to female parental investment [e.g. 14, 16]. Males of many species provide substantial parental investment or nuptial gifts [e.g. 22], and intrasexual competition for these direct benefits can result in strong sexual selection in females [23-25]. Thus, in order to better understand the drivers and consequences of sexual selection in females, we should direct attention to such species. We found that the evolutionary responses to altered OSR in females were well aligned with those typically observed in males in species with conventional mating systems [26, 27]. Females not only evolved to become more attractive to males, they also showed more rapid evolution of reproductive behavior than did males. Two recent experimental evolution studies of taxa with conventional sex-roles also found significant female trait evolution in response to sex ratio manipulation, without a measurable response in males [28, 29]. This accords with recent revaluations of theory suggesting that the strength of sexual selection in females may often have been grossly underestimated [2, 23]. We note that Fritzsche and Arnqvist [8] found that the opportunity for sexual selection was as high, or even higher, in female than male honey locust beetles.

The fact that we found few main effects of food treatment (Table 1) suggests that food resources are not generally exchangeable with male-provided resources in this system. In fact, females rarely if ever feed as adults, while males are observed to forage more frequently (Takakura 2004) and can mate more frequently when they do so (Fritzsche & Arnqvist 2015). We did find a significant interaction between sex ratio and feeding regime for the evolution of female courtship success (Figure 2, Table 1). Females from female-biased lines were very successful in achieving mating during their first courtship attempt under both feeding regimes. In contrast, females from male-biased lines achieved comparable success only if they evolved under low food availability. This suggests that copulations can to some extent compensate for food shortage: females evolved elevated courtship displays either when males were a limiting resource or when feeding regime likely slowed male remating rates even further.

We found no significant evolution of male investment in ejaculate size across treatments. Reference females did, however, produce less offspring after mating with males from lines that evolved under low food availability with a male-biased sex-ratio (Table S1, Figure S1). Interestingly, these males were also least attractive to females (Table 2, Figure S2). These results suggest that ejaculate composition evolved in response to food limitation, but that this effect is dependent upon the prevailing pattern of reproductive competition. In a similar experiment in the closely related *M. tonkineus*, males responded to elevated mate competition by

evolving increased ejaculate size and females benefitted from receiving large ejaculates [30]. Our results thus suggest that responses to reproductive competition in males can be multifaceted, presumably reflecting the complexity of ejaculate composition and the diversity of functions of various ejaculate components in seed beetles [31].

Our results indicate that the evolution of sexually selected traits in females need not signal fecundity, as is sometimes assumed [23]. Male honey locust beetles prefer to mate with females that perform more vigorous courtship displays [11, 13]. However, females with more intense courtship displays (i.e., from female-biased lines) neither produced more offspring nor lived longer (Table S2). Although this may reflect inferential limitations of our study, it is also consistent with the possibility of female 'sensory exploitation' of males [32] or indirect genetic benefits to males from mate choice (a 'sexy daughters' effect) [33, 34].

Our study [see also 30] is the first experimental evolution study of a sex-role reversed species. We found that female adaptations that increase female mating success evolved rapidly under conditions promoting strong reproductive competition among females. This led to the evolution of intensified sex-role reversal, as hypothesized by Darwin [1]. Behavioral traits evolving in females were not associated with significant fecundity benefits to males, suggesting that male mate choice may not be adaptive. Our study highlights the essential, but often overlooked, role that females play in mating system evolution [2, 23, 35].

Experimental procedures

Stock population

We established a large (> 500 individuals) laboratory stock population from a sample of a natural population of *M. dorsalis* (Inogashira Park, Tokyo, Japan; ≈ 3000 adults, June 2009). Beetles were reared on seeds of the honey locust *Gleditsia triacanthos* in climate-controlled chambers at 26 °C and 70 ± 10% relative humidity on a 16:8 light:dark cycle. All beetles were maintained in replicate 1L glass containers and were fed 20% sucrose solution, pollen, and water. Under these conditions, *M. dorsalis* has a generation time of approximately 6-7 weeks. We generated virgin individuals to start our experimental evolution lines, as well as to initiate each new generation of our experimental evolution, by isolating single beans with larvae in 24-well culture plates. We collected individual beetles as they hatched from the beans and kept them isolated until the onset of experiments.

Experimental evolution

We initiated 16 experimental populations, which were exposed to one of 4 treatment combinations (factorial 2 sex ratios × 2 food regimes design), using 4 replicate lines per treatment combination (N = 16 selection lines in total). The experimental lines were allowed to evolve in the laboratory for 19 generations. Lines were kept either at male-biased (125M:25F) or female-biased (25M:125F) sex-ratio conditions and beetles were either fed (20% sucrose solution and pollen) or were left unfed. Under female-biased conditions, female mating rate will be

relatively low, male mating rate will be high and reproductive competition between females will be intense. Food availability has profound effects in honey locust beetles, affecting for example life span, the cost of reproduction and male ejaculate size [12-13].

Every generation, 150 virgin adults per line were placed at the selected sex ratio in clean 1L glass jars containing a breeding substrate of *G. triacanthos* beans. Adults were allowed to mate and lay eggs in these jars for 2 weeks, and were then removed and discarded. Females cement their eggs to the inside of the jar; the larvae hatch after 5-7 days and seek out a bean, which they bore into to complete their development (3-4 weeks). "Fed" lines were provided with 20% sucrose solution, an ample supply of pollen granules (Bee Pollen Capsules, Manuka Health Ltd., New Zealand) and distilled water. "Unfed" lines were provided with distilled water only.

During the larval development period, prior to the emergence of new beetles from the beans, we distributed beans from each line individually in single wells of 24-well culture plates to allow the collection of virgin adults for the next generation. We note that, unlike in seed beetles with conventional sex roles, sex-specific variances in reproductive fitness are statistically indistinguishable in *M. dorsalis* [8]. Thus, the two sex ratio treatments used here show the same estimated effective population size ($N_e \sim 83$).

We terminated the experimental conditions at generation 19. To ensure that parental environmental effects, which can be transmitted for up to two generations in seed beetles [36], did not confound our results, the lines were maintained under common garden conditions of an equal sex ratio with no access to adult food for four subsequent generations prior to the assays described below.

Mating system assays: behavioral responses to experimental evolution

M. dorsalis shows sex-role reversal in courtship, in that females show active and extended courtship of males [11]. Typical courtships last from less than a minute to several minutes and consist of multiple repetitions of female turns [11, 13]. Males determine the outcome of courtship and reject their prospective mate in approximately 50% of female courtship attempts [11, 13].

In order to independently assay evolution of mating system variables in males and females, we performed assays where an individual from a selection line was paired with a randomly selected opposite-sex individual from a standard reference population (i.e., our outbred stock population). Each particular cross was replicated 7 - 8 times per line and sex. Virgin adults were collected from each of the 16 experimental evolution lines, as well as the reference population. Virgin adults were then paired on the 7th day following emergence. All individuals were weighed to the nearest 0.1mg prior to the assays (Sartorius® ME/SE analytical microbalance, Sartorius AG, Göttingen, Germany). Pairs were introduced together in 6cm plastic petri dishes and filmed for 30 minutes using a digital camera (Sony HDR CX250E).

After 30 minutes, pairs were separated, and each individual was re-weighed. In pairs that mated, the difference in male body weight before and after mating provides an accurate estimate of ejaculate weight [37]. We note that *M. dorsalis* males have a long refractory period (12-24 hrs) such that each pair mated either once only or not at all. Males were placed individually in 6cm plastic petri dishes and females in 12cm glass petri dishes containing 100g *G. triacanthos* beans for oviposition. We checked females and males daily until death to record life span and recorded the number of emerged offspring in each female dish after 8 weeks, when all offspring had emerged, as a measure of reproductive success.

We used video playbacks of all mating interactions to record courtship and mating behavior. Videos were analyzed by one observer blind to treatments. We recorded eight distinct aspects of male-female interactions, courtship behavior and mating: time to first encounter, time to the first courtship event, the number of turns performed in the first courtship bout, whether the first courtship resulted in mating or not, the total number of male mountings, time to mating, the total number of turns and whether courtship occurred or not. Here, 'turns' (number of times a female turns in front of a male to initiate mating) are a measure of courtship effort. In addition, we measured the mating duration in all trials where mating occurred.

Author Contributions: KF and GA conceived and designed the study, analysed and interpreted data and wrote the manuscript; KF and IB designed and performed

experiments; all authors reviewed and approved the manuscript. All data presented in this paper will be made available on dryad.

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Supplemental information:

- Table S1, S2
- Figure S1, S2
- Supplemental experimental procedures

Table 1: Response to experimental evolution in females.

Analyses of variance/covariance of the effects of selection regime (food treatment and sex ratio) on mating system parameters in assays where females from selection lines were paired with standard reference males.

Response variable	Source	ndf	ddf	*F*	*P*
Time to first encounter	Feeding regime	1	13	0.84	0.377
	Sex ratio	1	13	5.81	0.032
Time to first courtship*	Feeding regime	1	12	0.02	0.903
	Sex ratio	1	12	5.75	0.034
	Male weight	1	12	5.33	0.040
No. turns in 1st courtship bout*	Feeding regime	1	13	5.96	0.030
	Sex ratio	1	13	0.88	0.365
First courtship results in mating*	Feeding regime	1	12	3.78	0.076
	Sex ratio	1	12	9.55	0.009
	Feeding regime × Sex ratio	1	12	13.40	0.003
No. of mounting attempts*	Feeding regime	1	11	0.13	0.721
	Sex ratio	1	11	10.69	0.007
	Female weight	1	11	10.28	0.008
	Male weight	1	11	5.51	0.039
Time to mating*	Feeding regime	1	12	1.82	0.203
	Sex ratio	1	12	6.71	0.024
	Male weight	1	12	13.07	0.004
Total no. of turns*	Feeding regime	1	13	11.51	0.005
	Sex ratio	1	13	0.09	0.775
Courtship occurrence	Feeding regime	1	13	0.02	0.882
	Sex ratio	1	13	0.74	0.406
Ejaculate weight*	Feeding regime	1	13	0.22	0.644
	Sex ratio	1	13	1.01	0.332
Female body weight	Feeding regime	1	13	1.89	0.192
	Sex ratio	1	13	0.16	0.691
Mating duration*	Feeding regime	1	13	0.38	0.549
	Sex ratio	1	13	1.70	0.215

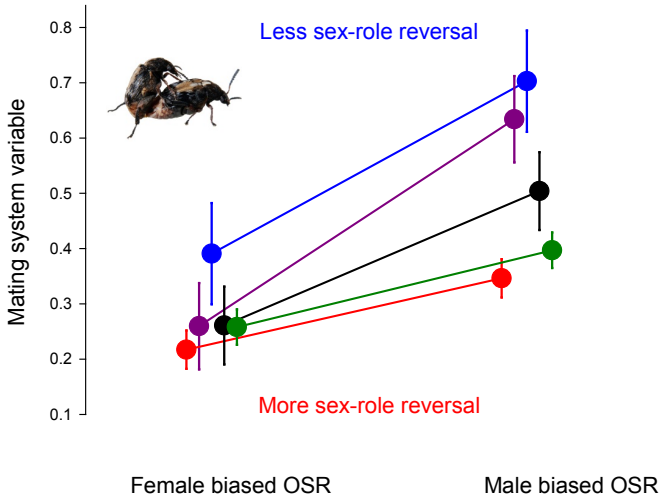
* These analyses excluded pairs that did not mate/court.

Table 2: Response to experimental evolution in males.

Analyses of variance/covariance of the effects of selection regime (food treatment and sex ratio) on mating system parameters in assays where males from selection lines were paired with standard reference females.

Response variable	Source	ndf	ddf	*F*	*P*
Time to first encounter	Feeding regime	1	13	1.68	0.217
	Sex ratio	1	13	2.82	0.117
Time to first courtship*	Feeding regime	1	13	4.87	0.046
	Sex ratio	1	13	3.60	0.080
No. turns in 1st courtship bout*	Feeding regime	1	13	0.07	0.790
	Sex ratio	1	13	2.06	0.175
First courtship results in mating*	Feeding regime	1	13	6.74	0.022
	Sex ratio	1	13	0.43	0.524
No. mounting attempts*	Feeding regime	1	13	3.48	0.085
	Sex ratio	1	13	0.31	0.586
Time to mating*	Feeding regime	1	13	2.89	0.113
	Sex ratio	1	13	0.89	0.364
Total no. turns*	Feeding regime	1	13	0.13	0.727
	Sex ratio	1	13	0.60	0.451
Courtship occurrence	Feeding regime	1	12	0.07	0.799
	Sex ratio	1	12	0.07	0.799
	Feeding regime × Sex ratio	1	12	11.64	0.005
Ejaculate weight*	Feeding regime	1	13	1.05	0.324
	Sex ratio	1	13	0.05	0.825
Male body weight	Feeding regime	1	13	1.49	0.243
	Sex ratio	1	13	0.27	0.611
Mating duration*	Feeding regime	1	13	0.03	0.869
	Sex ratio	1	13	0.11	0.748

* These analyses excluded pairs that did not mate/court.



386

387 **Fig. 1:** Females evolving under male-biased OSR became less sex-role reversed.

388 Mean evolutionary responses (±SEM) under male- or female-biased OSR for the five

389 behavioral variables that showed a significant effect of sex ratio, in assays where

390 selection line females were paired with standard reference males (Table 1): time to

391 1st encounter (red), time to first courtship (blue), time to mating (black), number of

392 mounts needed before mating occurred (purple) and the probability that the male

393 rejected the female in the first courtship attempt (green). High values are associated

394 with less pronounced sex-role reversal for all variables. For the purpose of

395 visualization, the first four variables are scaled here to fit within a range of 0 – 1

396 (scaling factors used are 0.005, 0.002, 0.001 and 0.2, respectively).

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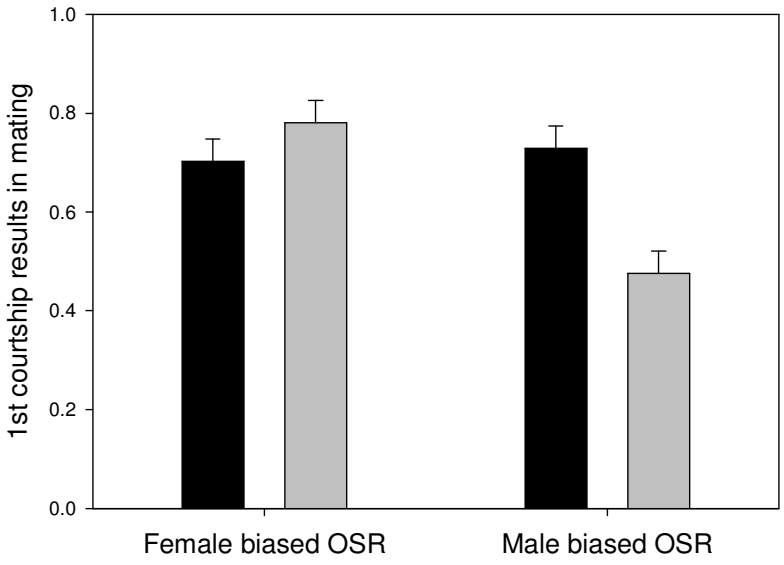

398

399 **Fig. 2:** Females evolving under male-biased OSR with abundant food became less

400 successful in converting courtship to mating.

401 Mean (±SEM) probability that the first courtship resulted in mating in assays where

402 selection line females were paired with standard reference males (Table 1) (fed:

403 grey bars; starved: black bars).

404

405